

LAIR-1, a Novel Inhibitory Receptor Expressed on Human Mononuclear Leukocytes

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Summary

In the present study, we describe a novel inhibitory receptor, leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1), that is constitutively expressed on the majority of human peripheral blood mononuclear leukocytes. LAIR-1 is a 32 kDa transmembrane glycoprotein with a single immunoglobulin-like domain and a cytoplasmic tail containing two immune receptor tyrosine-based inhibitory motifs. LAIR-1 recruits SHP-1 and SHP-2 phosphatases upon activation, and cross-linking of the LAIR-1 antigen on natural killer (NK) cells results in strong inhibition of NK cell-mediated cytotoxicity. Although it is structurally related to human killer cell inhibitory receptors, LAIR-1 does not appear to recognize human leukocyte antigen (HLA) class I molecules and thus represents a novel HLA class I-independent mechanism of NK cell regulation.

Introduction

Natural killer (NK) cells are a subpopulation of lymphocytes distinct from T and B cells. NK cells are capable of lysing transformed and virus-infected cells without apparent presensitization, and are considered important cellular components of innate immunity (reviewed by Trinchieri, 1989). The molecular interactions that trigger the cytolytic functions of NK cells are not completely understood. Monoclonal antibodies (MAbs) recognizing adhesion and signaling molecules, however, have been shown to induce NK cell-mediated cytotoxicity in vitro (Siliciano et al., 1985; Lanier et al., 1988b; Moretta et al., 1991; Shibuya et al., 1996).

In recent years, progress has been made in understanding the receptors responsible for inhibiting NK cell functions. The early observation that NK cells selectively kill targets that lack major histocompatibility complex (MHC) on their surface (Karre et al., 1986), eventually

led to the identification of inhibitory receptors present on NK cells that bind MHC expressed on the target cell. In humans, two separate families of inhibitory receptors have been identified on NK cells: killer cell inhibitory receptors (KIRs), which are immunoglobulin superfamily members (Colonna and Samaridis, 1995; D'Andrea et al., 1995; Wagtmann et al., 1995; Lanier and Phillips, 1996), and the CD94/NKG2 receptors of the C-type lectin superfamily (Lazetic et al., 1996; Phillips et al., 1996; Sivori et al., 1996). All of these inhibitory receptors possess cytoplasmic tails with immune receptor tyrosine-based inhibitory motifs (ITIMs) (Thomas, 1995). Upon phosphorylation, cytoplasmic ITIMs bind the SH2 domains of certain phosphatases, leading to down-regulation of cell activation (Scharenberg and Kinet, 1996).

To identify molecules important in the regulation of NK cell cytotoxicity, we immunized mice with human NK cells and screened for MAbs that were capable of inhibiting NK cell mediated cytotoxicity. A MAb, designated DX26, that when cross-linked delivered a negative signal to NK cells, was identified. The antigen recognized by DX26 MAb (designated leukocyte-associated immunoglobulin-like receptor-1 [LAIR-1]) is a member of the immunoglobulin superfamily, with homology to KIRs, and possesses two ITIMs in its cytoplasmic domain. LAIR-1, however, does not appear to recognize class I human leukocyte antigens (HLA) and thus represents a novel (HLA-independent) inhibitory molecule that may play an important role in the regulation of NK cell function.

Results

DX26 Identifies an Inhibitory Receptor on NK Cells

To identify molecules that are involved in negative signaling, mice were immunized with a human NK cell clone and antibodies were screened for their capacity to inhibit NK cell-mediated lysis of Fc-receptor (FcR)-bearing targets. A MAb, designated DX26, was isolated that efficiently inhibited the NK cell-mediated cytotoxicity of the human Fc γ RII (CD32) transfected Epstein-Barr virus (EBV)-transformed B cell line, 721.221/CD32 (Figure 1). Cytotoxicity of the FcR-negative parent line, 721.221, however, was not affected by DX26 MAb, indicating that FcR cross-linking of LAIR-1 (the antigen recognized by DX26 MAb) was required to deliver the negative signal (Figure 1). Further evidence for the inhibitory signal-transducing capabilities of LAIR-1 was obtained using the FcR-expressing mouse mastocytoma cell line, P815. Many NK cell clones lyse P815 in the presence of MAbs against positive signal molecules, such as CD2, CD16, CD69, and DNAM-1 (Lanier et al., 1997). Antibody-mediated NK cell lysis of P815 was also inhibited by DX26 MAb, indicating that cross-linking of LAIR-1 led to inhibition of cytotoxicity, even in the presence of strong positive signals (Figure 2).

Since the majority of resting NK cells express LAIR-1, experiments were undertaken to determine whether

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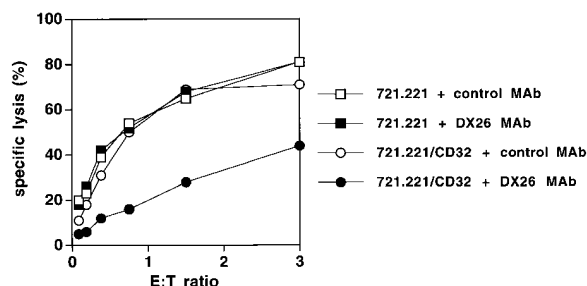


Figure 1. DX26 MAb Inhibits NK Cell Cytotoxicity When Cross-Linked by FcR on the Target Cell

NK clone KS279.p1 was assayed for lysis of 721.221 target cells or 721.221 transfectants expressing the human FcγRII (721/CD32) in the presence of DX26 MAb or control IgG1. DX26 MAb and control IgG1 were used at 10 μg/ml. Data are representative of experiments performed with several NK cell clones.

LAIR-1 functions as an inhibitory receptor on freshly isolated NK cells. Resting NK cells were purified from peripheral blood to greater than 90% purity and assayed for cytolysis of anti-CD16 MAb-coated P815 in the presence or absence of DX26 MAb. As was observed with NK cell clones, freshly isolated NK cell cytolysis of anti-CD16 coated P815 was significantly inhibited by the presence of DX26 MAb (Figure 3A). Cytolysis of the NK-sensitive tumor cell line K562 by NK cells in freshly isolated peripheral blood mononuclear cells (PBMCs) was also inhibited by DX26 MAb (Figure 3B). DX26 F(ab)₂ fragments did not affect killing of K562, which further substantiates the necessity for LAIR-1 to be cross-linked in order to generate a negative signal in NK cells. Likewise, NK cell cytotoxicity against a panel of FcR-negative tumor cells was unaffected by the presence of the DX26 MAb (data not shown).

LAIR-1 Is a Broadly Expressed Leukocyte Antigen

Phenotypic analysis of human peripheral blood lymphocytes demonstrated that LAIR-1 is a widely expressed molecule on peripheral blood leukocytes. LAIR-1 is prominently expressed on normal peripheral blood CD3⁺CD4⁺ T cells (70%–80%), CD3⁺CD8⁺ T cells (80%–90%), CD3⁺CD56⁺ NK cells (95%–100%), CD3⁺CD19⁺

B cells (80%–90%), and CD3⁺CD14⁺ monocytes (99%–100%) (Figures 4A–4C). LAIR-1, however, was not expressed on peripheral blood granulocytes, platelets, or red blood cells (data not shown). The majority of human fetal thymocytes, also expressed LAIR-1 (Figure 4D), as well as all NK cell and T cell clones and all T cell tumor cell lines (Table 1). The long-term cultured NK lines, NKL and NK92, were negative for LAIR-1, as were EBV-transformed B cell lines, the B cell tumor Daudi, the NK-like tumor cell line YT, and several nonhematopoietic cell lines (Table 1).

cDNA Cloning of LAIR-1

To identify a LAIR-1-encoding cDNA, 293T cells were transfected with a cDNA library prepared from a polyclonal human NK cell line (Lanier et al., 1994), and expression cloning using flow cytometry was performed. As shown in Figure 5A, a single cDNA that encoded a protein recognized by the DX26 MAb was obtained. The LAIR-1 cDNA was 1728 bp, containing an 864 bp open reading frame encoding a type I membrane protein with a 21 amino acid (aa) leader sequence, a 142 aa extracellular domain, a 23 aa transmembrane domain, and a 101 aa cytoplasmic region (Figures 5B and 5C). One pair of cysteines in the extracellular domain generates a single immunoglobulin-like domain, thus classifying LAIR-1 as a member of the immunoglobulin superfamily. The extracellular domain had one potential N-linked glycosylation site. The predicted molecular weight of the LAIR-1 polypeptide is 32 kDa. The cytoplasmic domain contained the amino acid sequences VTAAQL and ITAAV, spaced by 24 aa, which fit the consensus sequences for ITIMs (V/IxYxxxL/V) (D'Ambrosio et al., 1995). Using fluorescence in situ hybridization, the LAIR-1 gene was mapped to chromosome 19q13.4.

Northern blot analysis of PBMCs showed two predominant transcripts of approximately 1.7 and 3 kb (Figure 6). Transcripts were found in human monocytes, Jurkat, U937, and human NK cell clones, but not in LAIR-negative cell lines, such as the EBV-transformed B cell line JY (Figure 6). Southern blot analysis of human genomic DNA demonstrated a relatively simple hybridization pattern for LAIR-1 (data not shown). This uncomplicated

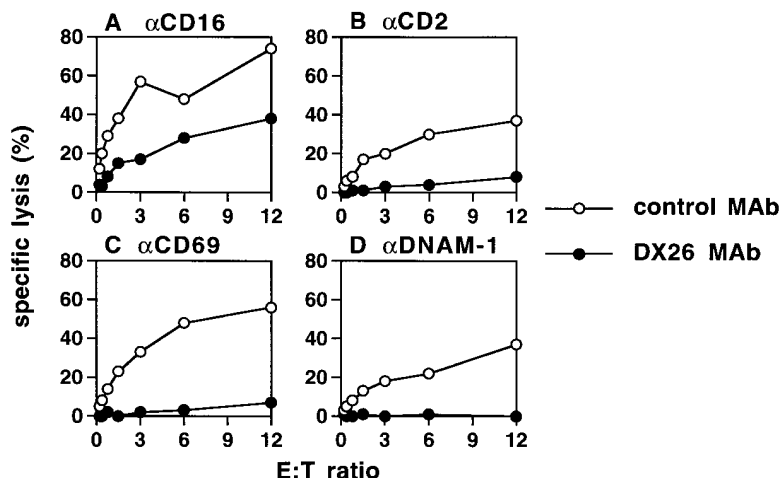


Figure 2. DX26 MAb Inhibits NK Cell Cytotoxicity in the Presence of Cross-Linking MAb to Positive Signaling Structures

NK clone KS279.p1 was assayed for lysis of P815 target cells, when stimulated with 5 μg/ml MAb against CD16 (A), CD2 (B), CD69 (C), or DNAM-1 (D) in the presence of DX26 MAb or control IgG1. DX26 MAb and control IgG1 were used at 10 μg/ml. Data are representative of experiments performed with several NK cell clones.

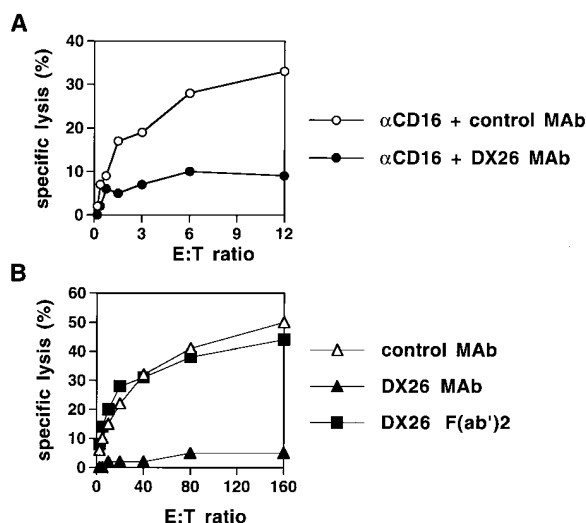


Figure 3. DX26 MAb Inhibits Cytolytic Function of Resting NK Cells (A) NK cells were purified from human peripheral blood and assayed for lysis of P815 cells when stimulated with 5 μ g/ml anti-CD16 in the presence of 10 μ g/ml control IgG1 or DX26 MAb. Data are representative of three independent experiments with cells from different donors. (B) Freshly isolated PBMCs were used as effector cells and assayed for spontaneous killing of K562 cells in the presence of 10 μ g/ml control IgG1, DX26 MAb, or DX26 F(ab')₂ fragments. Data are representative of experiments with cells from four different donors.

genomic organization suggests that the LAIR-1 probe hybridizes with a single or relatively few genes.

Biochemistry of LAIR-1

To study the biochemical features of LAIR-1, the protein was immunoprecipitated with DX26 MAb from ¹²⁵I-labeled lysates of 293T cells transfected with the LAIR-1 cDNA and two peripheral blood human NK cell clones. LAIR-1 precipitated from LAIR-1-transfected cells was a ~40 kDa monomer when analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under both nonreducing and reducing conditions (Figure 7). The mobility of LAIR-1 decreased from ~40 to ~32 kDa after treatment with N-glycosidase F, in agreement with the predicted protein size and one N-linked glycosylation site (Figure 7). Immunoprecipitation with DX26 MAb from lysates of two human NK cell clones revealed, in addition to LAIR-1 at 40 kDa, a smaller protein migrating at ~32 kDa (Figure 7). This smaller protein also decreased in size after deglycosylation. Whether this represents a distinct LAIR-1 isoform or a proteolytic cleavage product of LAIR-1 is unknown.

LAIR-1 Binds SHP-1 and SHP-2

The existence of two consensus sequences for ITIMs within the cytoplasmic domain of LAIR-1 suggested that the generation of an inhibitory signal in NK cells was manifested by the recruitment of SH2-containing tyrosine phosphatases-1 and/or -2 (SHP-1 and/or SHP-2). To determine whether LAIR-1 was capable of binding protein tyrosine phosphatases, a NK cell clone was stimulated with pervanadate (an inhibitor of protein tyrosine phosphatases that induces tyrosine phosphorylation

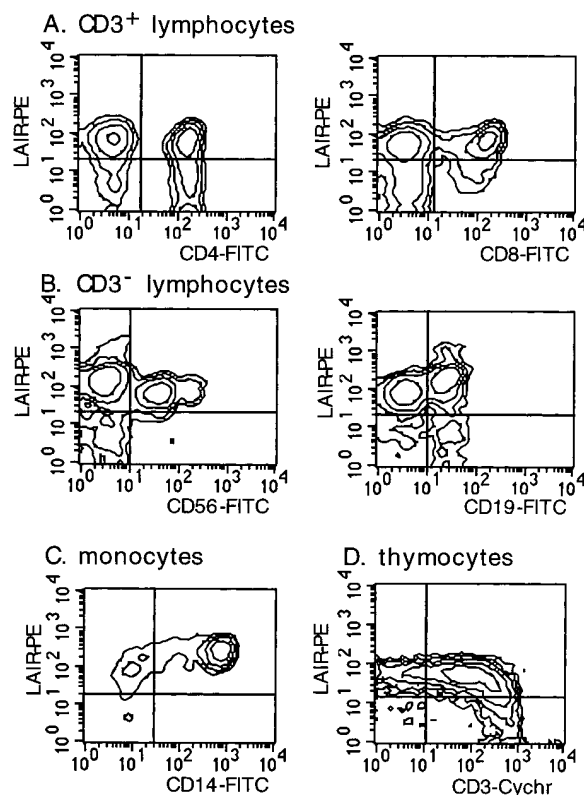


Figure 4. LAIR-1 Expression on Peripheral Blood Leukocytes and Fetal Thymocytes

(A–C) PBMCs were stained with DX26 MAb and visualized by phycoerythrin (PE)-conjugated goat-anti-mouse-IgG; CyChrome-conjugated CD3 MAb; and fluorescein isothiocyanate (FITC)-conjugated CD4, CD8, CD56, CD19, or CD14 MAb. Cells were analyzed on a FACScan and gated for CD3⁺ lymphocytes (A), CD3⁻ lymphocytes (B), or monocytes (C) on the basis of forward and side scatter profiles and anti-CD3 MAb staining.

(D) Human fetal thymocytes were stained with DX26 MAb visualized by PE-conjugated goat anti-mouse IgG and CyChrome-conjugated anti-CD3 MAb.

Markers were set using appropriate isotype control antibodies. Data are representative of three independent experiments.

(O'Shea et al., 1992), lysed, and immunoprecipitated with DX26 MAb. Immunoprecipitates were then analyzed by Western blot using antibodies specific for SHP-1 and SHP-2. As shown in Figure 8, both SHP-1 and SHP-2 associated with tyrosine-phosphorylated LAIR-1. These results suggest that recruitment of SHP-1 and SHP-2 may be involved in mediating the negative signal transduced via engagement of the LAIR-1 molecule.

Discussion

Regulation of immune responses is determined by the balance of positive signals to initiate an immune response and inhibitory mechanisms to prevent excessive inflammatory responses and autoimmunity. Here we describe a novel inhibitory receptor, LAIR-1, that may function to down-regulate immune responses. LAIR-1 is a novel member of the immunoglobulin superfamily that is expressed on the majority of peripheral blood

mononuclear leukocytes. Cross-linking of LAIR-1 on human NK cells delivers a potent inhibitory signal that is capable of inhibiting target cell lysis by both resting and activated NK cells in vitro.

LAIR-1 is structurally related to several other inhibitory immunoglobulin superfamily members, including human KIRs (Colonna and Samaridis, 1995; D'Andrea et al., 1995; Wagtmann et al., 1995), human FcR α (Maliszewski et al., 1990), bovine Fc γ RII (Zhang et al., 1995), mouse gp49 (Arm et al., 1991; Rojo et al., 1996), and mouse gp91 or paired immunoglobulin-like receptors (Hayami et al., 1997; Kubagawa et al., 1997). The overall homology of LAIR-1 with these proteins is less than 30%. LAIR-1, however, shows slightly higher homology (32%) to members of a recently identified novel group of genes designated immunoglobulin-like transcripts (ILTs) (Cella et al., 1997; Samaridis and Colonna, 1997). LAIR-1, KIRs, ILTs, and FcR α all are localized to the same human chromosome region, 19q13.4, suggesting that these molecules evolved from a common ancestral gene.

Consistent with the inhibitory signal given to NK cells, the cytoplasmic domain of LAIR-1 contains two tyrosine-based inhibitory motifs (VTYAQL and ITYAAV). In the human KIRs, similar ITIMs were found to recruit phosphatases (primarily SHP-1) upon tyrosine phosphorylation and thereby to inhibit positive signal transduction via other receptors (Burshtyn et al., 1996; Campbell et al., 1996; Fry et al., 1996; Olcese et al., 1996; Scharenberg and Kinet, 1996). Indeed, activation of NK cells with sodium pervanadate resulted in the binding of both SHP-1 and SHP-2 phosphatases to LAIR-1. This suggests that the binding of these phosphatases to LAIR-1 might lead to inhibition of downstream molecular events associated with cellular activation.

LAIR-1 may be a member of a novel family of highly related receptors, similar to the situation with ILTs, gp49, and KIRs. Northern blot analysis revealed at least two distinctive mRNA transcripts, and immunoprecipita-

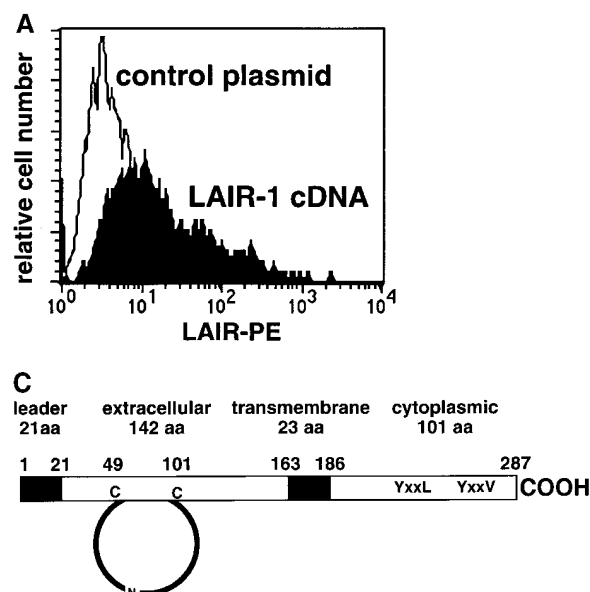


Figure 5. cDNA Cloning of *LAIR-1*

tions with DX26 MAb identified two proteins with slightly different molecular weights after deglycosylation. We have recently identified, by colony hybridization of a T cell-derived cDNA library, a putative family member of LAIR-1, which we have designated LAIR-2 (EMBL/GenBank/DBJ accession number AF013250). LAIR-2 contains one immunoglobulin-like domain, which is 84% homologous to LAIR-1; however, it lacks both a transmembrane and a cytoplasmic domain, suggesting that LAIR-2 is a secreted protein (data not shown). Further studies are required to delineate the functions of LAIR-2 as well as to define other possible LAIR-1 family members.

We have not yet defined the ligand(s) for LAIR-1. Since

Table 1. Expression of LAIR on Human Tumor Cell Lines

Cell Line	Type	Mean Fluorescence Intensity	
		Control IgG1	DX26 MAb
HUT78	T cell tumor	<5	25.8
Peer	T cell tumor	<5	29.1
Molt4	T cell tumor	<5	30.7
CEM	T cell tumor	<5	92.7
Jurkat	T cell tumor	<5	47.1
HL60	Promyeloid tumor	<5	46.9
U937	Myeloid tumor	<5	49.5
721.221	EBV B cell	<5	<5
JY	EBV B cell	<5	<5
Daudi	B cell tumor	<5	<5
YT	NK cell tumor	<5	<5
NKL	NK cell line	<5	<5
NK92	NK cell line	<5	<5
Colo205	Colon carcinoma	<5	<5
293T	Embryonic kidney	<5	<5
PA-1	Teratocarcinoma	<5	<5
FO-1	Melanoma	<5	<5

Cells were stained with control IgG1 or DX26 MAb and PE-conjugated goat-anti-mouse-IgG as a second step. Cells were analyzed on a FACScan. Data are representative of three independent experiments.

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AAAGGCTGCAGAGTCTGTCTTGCATGGTGCCTCAGGCCAGGCTGCACTGCTGGG 59
ACCTGGGCGCATGTCTCCCCACCCACCGCCTCCTGGGCTAGTGCTCTGCCTGGCCCA 118
      M S P H P T A L L G L V L C L A Q
GACCATCCACACGCAGGAGGAAGATCTGCCAGACCCCTCCATCTCGGCTGAGCCAGGCA 177
      T I H T Q E E D L P R P S I S A E P G
CCGTGATCCCCCTGGGAGCCATGTGACTTTCGTGTGCCGGGGCCCGTTGGGGTTCAA 236
      T V I P L G S H V T F V C R G P V G V Q
ACATTCCGCCTGGAGAGGAGAGTAGATCCACATACAATGATACTGAAGATGTGTCTCA 295
      T F R L E R E S R S T Y N D T E D V S Q
AGCTAGTCCATCTGAGTCAGAGGCCAGATTCCGCATTGACTCAGTAAGTGAAGGAAATG 354
      A S P S E S E A R F R I D S V S E G N
CCGGCCCTTATCGTGTGATCTATTATAAGCCCCCTAAATGGTCTGAGCAGAGTGACTAC 413
      A G P Y R C I Y Y K P P K W S E Q S D Y
CTGGAGCTGTGGTGAAGAAACCTCTGGAGGCCCGGACTCCCGGACACAGAGCCCGG 472
      L E L L V K E T S G G P D S P D T E P G
CTCCTCAGCTGGACCCACGCAGAGGCCGTCGGACAACAGTCACAATGAGCATGCACCTG 531
      S S A G P T Q R P S D N S H N E H A P
CTTCCCAAGGCTGAAAGCTGAGCATCTGTATATTCTCATCGGGGTCTCAGTGGTCTTC 590
      A S Q G L K A E H L Y I L I G V S V V F
CTCTTCTGTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT 649
      L F C L L L L V L F C L H R Q N Q I K Q
GGGGCCCCCAGAAGCAGGACGAGGAGCAGAAGCCACAGCAGAGGCTGACCTGGGCTG 708
      G P P R S K D E E Q K P Q Q R P D L A
TTGATGTCTTAGAGAGGACAGCAGACAAGGCCACAGTCAATGGACTTCCCTGAGAAGGAC 767
      V D V L E R T A D K A T V N G L P E K D
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      R E T D T S A L A A G S S Q E V T Y A Q
GCTGGACCACTGGGCCCTCACACAGAGGACAGCCCGGGCTGTGTCCCCACAGTCCACAA 885
      L D H W A L T Q R T A R A V S P Q S T
AGCCCATGGCCGAGTCCATCAGTATGCAGCCGTTGCCAGACACTGACCCCATACCCAC 944
      K P M A E S I T Y A A V A R H *
CTGGCCTCTGCACCTGAGGGTAGAAAGTCACTCTAGGAAAAGCCTGAAGCAGCCATTG 1003
GAAGGCTTCTGTGGATTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT 1062
ACAAGAGCTGGAGACTGGAGGTTTCTAACCAGCATCCAGAAGGTTCTGTTAGCCAGGTGG 1121
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CAGCTACAGTTCCCTCTGGCTGTTTCTAGAGACCCAGCTTTATTCACCTGACTGTTCCAG 1239
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(Figure 5 continued from previous page)

(A) LAIR-1 expression on 293T cells transfected with the pJFE14 vector containing an irrelevant insert or *LAIR-1* cDNA.

(B) Nucleotide and predicted amino acid sequences of LAIR-1. The leader sequence and putative transmembrane domain are underlined. The potential N-linked glycosylation site in the extracellular domain is circled. The ITIM sequences in the cytoplasmic domain are indicated by underlining and bold print. These sequence data are available from EMBL/GenBank/DBJ under accession number AF013249.

(C) Schematic diagram of the LAIR-1 protein. The diagram indicates leader peptide, extracellular, transmembrane, and cytoplasmic regions and the pair of cysteine residues in the extracellular domain potentially able to participate in intrachain disulfide bonding for the formation of an immunoglobulin-like domain.

the DX26 MAb does not interfere with HLA class I recognition by a large panel of NK clones (data not shown), it seems unlikely that LAIR-1 recognizes HLA class I molecules. Furthermore, since LAIR-1 is broadly expressed on the majority of mononuclear leukocytes, its ligand(s) may demonstrate a more restricted expression. Experiments are presently underway using LAIR-1 fusion proteins to probe various tissues and cell types for potential ligands for this inhibitory receptor.

At present, we have studied the function of LAIR-1 only on peripheral blood NK cells. DX26 MAb also stains peripheral blood B cells, T cells, and monocytes as well as the majority of thymocytes. The functional significance of the molecules recognized by DX26 MAb on these other mononuclear leukocytes remains to be determined. However, it is reasonable to predict that

LAIR-1, or closely related family members, will be found to provide a mechanism of regulation in a variety of immune interactions.

Experimental Procedures

Cells

Peripheral blood from healthy donors was purchased from Stanford Blood Center (Stanford, CA). PBMCs were isolated by Ficoll-Hypaque centrifugation. Resting NK cells were purified from PBMCs by magnetic bead depletion as described (Phillips et al., 1992).

NK clones were established as described (Phillips et al., 1991) and maintained in culture using the method of Yssel et al. (1984).

The HLA class I-deficient EBV-transformed lymphoblastoid cell line 721.221 has been described (Shimizu and DeMars, 1989). DT287 is a stable transfectant of 721.221, expressing human FcγRII (CD32), generated in our laboratory. 293 cells expressing SV40 large T antigen (293T) were generously provided by T. Kitamura (DNAX). P815,

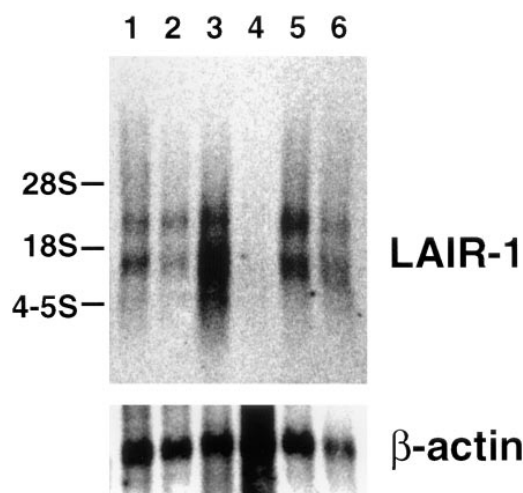


Figure 6. Expression of LAIR-1 mRNA

Total RNA of human monocytes (lane 1), Jurkat (lane 2), U937 (lane 3), JY (lane 4), and two different human NK cell clones (lanes 5 and 6) was analyzed for LAIR transcripts using a 32 P-labeled LAIR-1 probe (top). As a control for the amount of RNA in each lane, hybridization with a β -actin probe of the same blot is also shown (bottom).

HUT78, Peer, Molt4, CEM, Jurkat, HL60, U937, JY, Daudi, Colo205, PA-1, and K562 were obtained from the American Type Culture Collection (Rockville, MD). YT-2C2 is a NK-like tumor cell line that was kindly provided by K. Smith (Yodoi et al., 1985). FO-1 is a human melanoma cell line that was a kind gift of S. Ferrone (D'Urso et al., 1991). NKL is an NK cell line generously provided by M. Robertson (Dana-Farber, Boston, MA) (Robertson et al., 1996). The NK92 cell line was obtained from H. G. Klingemann (Gong et al., 1994).

Antibodies

The DX26 (immunoglobulin G1 [IgG1]) α -producing hybridoma was generated by fusing the Sp2/0 myeloma cell line with splenocytes from a BALB/c mouse immunized with a human NK clone. Antibodies against DNAM-1 (DX11) (Shibuya et al., 1996) and CD69 (L78) (Testi et al., 1989) have been described. DX10 (mouse IgG1) is a CD2 MAb that was generated in our laboratory. All other MAbs were generously provided by Becton Dickinson Immunocytometry Systems (San Jose, CA).

Cytotoxicity Assay

Cell lines were labeled with 51 Cr and used as targets in a 4 hr radioisotope release assay as described (Lanier et al., 1983). Data are expressed as the mean of triplicate cultures. Spontaneous radioisotope release did not exceed 10% of maximum release as determined by lysis of target cells with 10% Triton-X 100. The percentage specific lysis was calculated as (cpm specific 51 Cr release – cpm spontaneous 51 Cr release)/(cpm maximum 51 Cr release – cpm spontaneous 51 Cr release) \times 100.

Expression Cloning

A cDNA library was constructed from an interleukin-2-dependent polyclonal NK line, as described (Lanier et al., 1994). cDNA cloning by transient expression in 293T cells was performed as described (Aruffo and Seed, 1987), with modifications (Lanier et al., 1994).

RNA and DNA Analysis

cDNA was sequenced by using the dideoxy termination technique with a Sequenase 2.0 kit (United States Biochemical, Cleveland, OH) and by using an automated nucleic acid sequencer (Applied Biosystems, Foster City, CA).

The 1.7 kb LAIR-1 cDNA was released from the plasmid by digestion with NotII and BstXI restriction enzymes, labeled using random hexamer primers (Feinberg and Vogelstein, 1983), and used as a

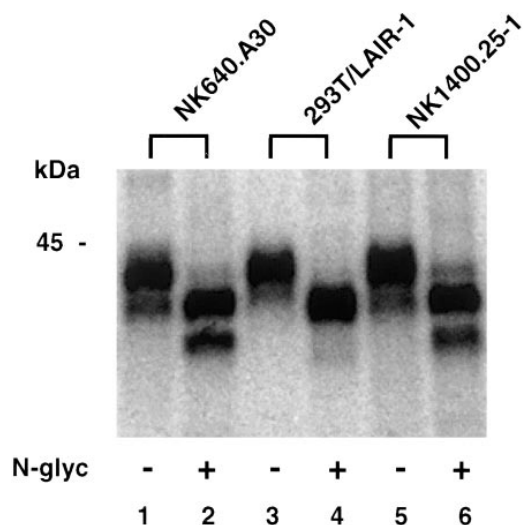


Figure 7. LAIR-1 Is a 32 kDa Protein Expressed as a Monomer

125 I-labeled 293T cells, transfected with the LAIR-1 encoding cDNA (lane 3 and 4) and two different human NK cell clones (lanes 1, 2, 5, and 6) were lysed, and LAIR-1 antigen was immunoprecipitated with DX26 MAb. The immunoprecipitates were treated with N-glycosidase where indicated and analyzed by SDS-PAGE under nonreducing conditions. Analysis under reducing conditions resulted in an identical migration of the proteins (data not shown).

probe in Northern and Southern blot analyses. Total RNA was isolated with RNA-STAT-60 (Teltest 'B', Friendswood, TX). RNA and DNA were separated by electrophoresis in agarose gels, transferred to membranes, and hybridized with the 32 P-labeled probes at 65°C in Quickhyb (Stratagene Cloning Systems, La Jolla, CA) according to the instructions of the manufacturer. Radioactivity was measured using a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

The chromosomal localization of the LAIR-1 gene was determined using a biotinylated LAIR-1 probe for in situ hybridization to metaphases from two normal males as described (Callen et al., 1990).

Biochemistry

To characterize biochemically the antigens recognized by DX26 MAb, cells were labeled with 125 I and antigens immunoprecipitated

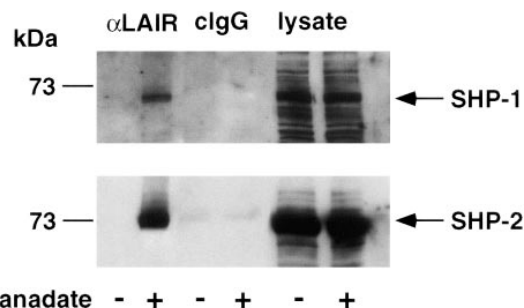


Figure 8. SHP-1 and SHP-2 Are Associated with LAIR-1 in NK Cells upon Pervanadate Stimulation

A human NK cell clone was incubated in the absence or presence of pervanadate for 10 min at 37°C. Cells were lysed and LAIR-1 proteins were immunoprecipitated with DX26 MAb. As a control, immune precipitation with control IgG is shown. Immunoprecipitates were separated by SDS-PAGE and blotted with SHP-1 (top) and SHP-2 (bottom) antibodies. As a control for the presence of SHP-1 and SHP-2, whole cell lysates were run on the same gel (right lanes).

by a procedure described previously (Lanier et al., 1988a). Immuno-precipitates were separated by SDS-PAGE and visualized by exposure to a phosphorimager screen.

To demonstrate association of phosphatases with phosphorylated LAIR-1, NK cells were stimulated with 100 mM sodium pervanadate for 10 min at 37°C (O'Shea et al., 1992). Cells were lysed in 1% NP40 in the presence of phosphatase inhibitors as described (Phillips et al., 1996). LAIR-1 protein was immunoprecipitated by DX26 MAb and protein G-sepharose (Pharmacia, Alameda, CA). Immunoprecipitates were separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA). The membranes were blocked and incubated with polyclonal rabbit antibodies against SHP-1 and SHP-2 (C18 and C19, Santa Cruz Laboratories), followed by horseradish peroxidase conjugated anti-rabbit immunoglobulin (Amersham, Arlington Heights, IL). Proteins were detected by enhanced chemiluminescence (Supersignal Substrate, Pierce, IL).

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